Long Telomeres Bypass the Requirement for Telomere Maintenance in Human Tumorigenesis

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Long Telomeres Bypass the Requirement for Telomere Maintenance in Human Tumorigenesis

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SUMMARY

Despite the importance of telomere maintenance in cancer cell survival via the elongation of telomeres by telomerase reverse transcriptase (TERT) or alternative lengthening of telomeres (ALT), it had not been tested directly whether telomere maintenance is dispensable for human tumorigenesis. We engineered human tumor cells containing loxP-flanked *hTERT* to enable extensive telomere elongation prior to complete *hTERT* excision. Despite unabated telomere erosion, *hTERT*-excised cells formed tumors in mice and proliferated in vitro for up to 1 year. Telomerase reactivation or ALT was not observed, and the eventual loss of telomeric signal coincided with loss of tumorigenic potential and cell viability. Crisis was averted via the reintroduction of active but not inactive hTERT. Thus, telomere maintenance is dispensable for human tumorigenesis when telomere reserves are long. Yet, despite telomere instability and the presence of oncogenic *RAS*, human tumors remain susceptible to crisis induced by critically short telomeres.

INTRODUCTION

The limited in vitro life span of normal human cells, referred to as the Hayflick limit, cellular senescence, or mortality stage 1 (M1), was first described in 1961 (Hayflick, 1973). The temporal onset of senescence is correlated tightly to telomere length (Allsopp et al., 1992; Harley et al., 1990), and is bypassed by expression of the telomerase reverse transcriptase *hTERT* (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Transformation via factors such as SV40 T antigen lead to life span extension beyond M1; however, cells acquire genetic instability and eventually undergo apoptosis, referred to as M2 or crisis (Wright et al., 1989). Further, the discovery that tumor cells possessed shorter telomeres compared with normal tissues suggested that telomere maintenance was required to avert crisis during tumorigenesis (de Lange et al., 1990; Hastie et al., 1990). This hypothesis was borne out in SV40-transformed human cells, in which rare clones that acquired telomerase activity survived the genetic instability and cell death that accompany crisis (Counter et al., 1992). In fact, enforced expression of *TERT* in combination with oncogenic *RAS* and the SV40 early region (ER) elicits tumorigenic conversion of fibroblast, kidney epithelial, and mammary epithelial cells (Elenbaas et al., 2001; Hahn et al., 1999a, 2002). Thus, the acquisition of telomerase activity appears essential for immortality in many normal and cancer cell types.

While mice have proven a useful model system in which to study cancer, the response to a critically short telomere differs markedly between mice and humans (for review, see Smogorzewska and de Lange, 2002). Another critical difference between mice and humans is that many human tumor cell types possess a subset of telomeres that are already critically short (Capper et al., 2007; Xu and Blackburn, 2007), whereas laboratory murine strains typically possess much longer average telomere lengths (Hemann and Greider, 2000). For example, inhibition of telomerase reverse transcriptase (TERT) in human tumor lines induces cell death almost immediately, confounding the ability to distinguish the role of TERT in cell viability independent of telomere maintenance (Hahn et al., 1999b; Zhang et al., 1999). Thus, an important unresolved question is whether TERT, or indeed any mechanism of telomere maintenance, is essential for human tumorigenesis.

To address this question, we engineered a human tumor line in which telomere length and *hTERT* expression could be controlled genetically and temporally. We employed the

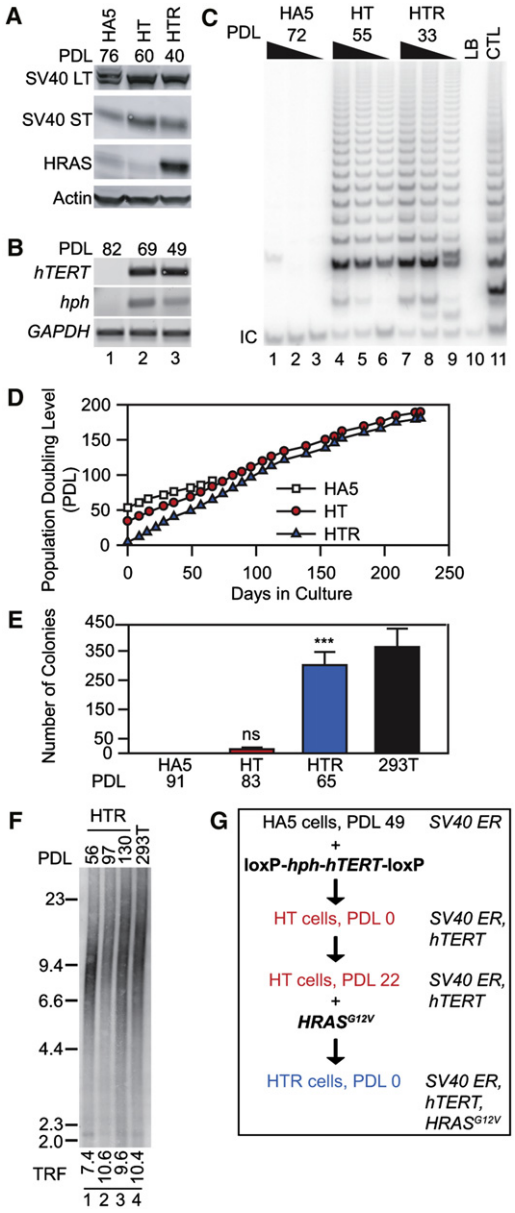


Figure 1. An *hTERT*-Excisable Tumorigenic Cell Line

(A) Western analysis of whole-cell lysates (50 μg) from HA5, HT (HA5 + TERT), and HTR (HT + RAS) cells at indicated population-doubling level (PDL).

(B) RT-PCR analysis of *hTERT*, *hph*, and *GAPDH* at indicated PDL.

(C) Analysis of telomerase activity of cell lysates (200, 100, 50 ng) at indicated PDL. LB, negative buffer control; CTL, HeLa cell lysate-positive control; IC, internal control PCR product.

(D) Replicative life span of HA5, HT, and HTR cells. HT or HTR cells were immortal.

(E) Anchorage-independent colony growth at indicated PDL (n = 3). 293T cells were a positive control for colony formation. Statistical significance between HA5 (no colonies formed) and HT or HTR cell lines as indicated (n = 3, ***$p < 0.001$; ns, $p > 0.05$, $power^{(1-\beta\ err\ prob)} > 0.99$, $\alpha^{actual} = 0.05$, two tailed).

(F) TRF analysis of average telomere length at increasing PDL. 293T cells were included as a control. Weighted mean telomere lengths (kbp) are indicated below each lane.

(G) Schematic of elements introduced into HA5 cells, at indicated PDL.

Error bars illustrate the standard deviation of the mean, using the number of replicates as indicated.

Cre-loxP system, which enables stringent and reversible control of *hTERT* in primary human cells to generate human tumor cells with long telomeres from which *hTERT* could be excised (Cascio, 2001; Jaiswal et al., 2007; Steinert et al., 2000; Ungrin and Harrington, 2006). The results demonstrated unequivocally that TERT is dispensable for human tumorigenesis and cell viability when telomeres are long. However, despite the continuous presence of *RAS* and SV40, induction of endogenous telomerase or other telomere maintenance mechanisms (e.g., ALT) was not observed, and the cells eventually succumbed to telomere-induced crisis.

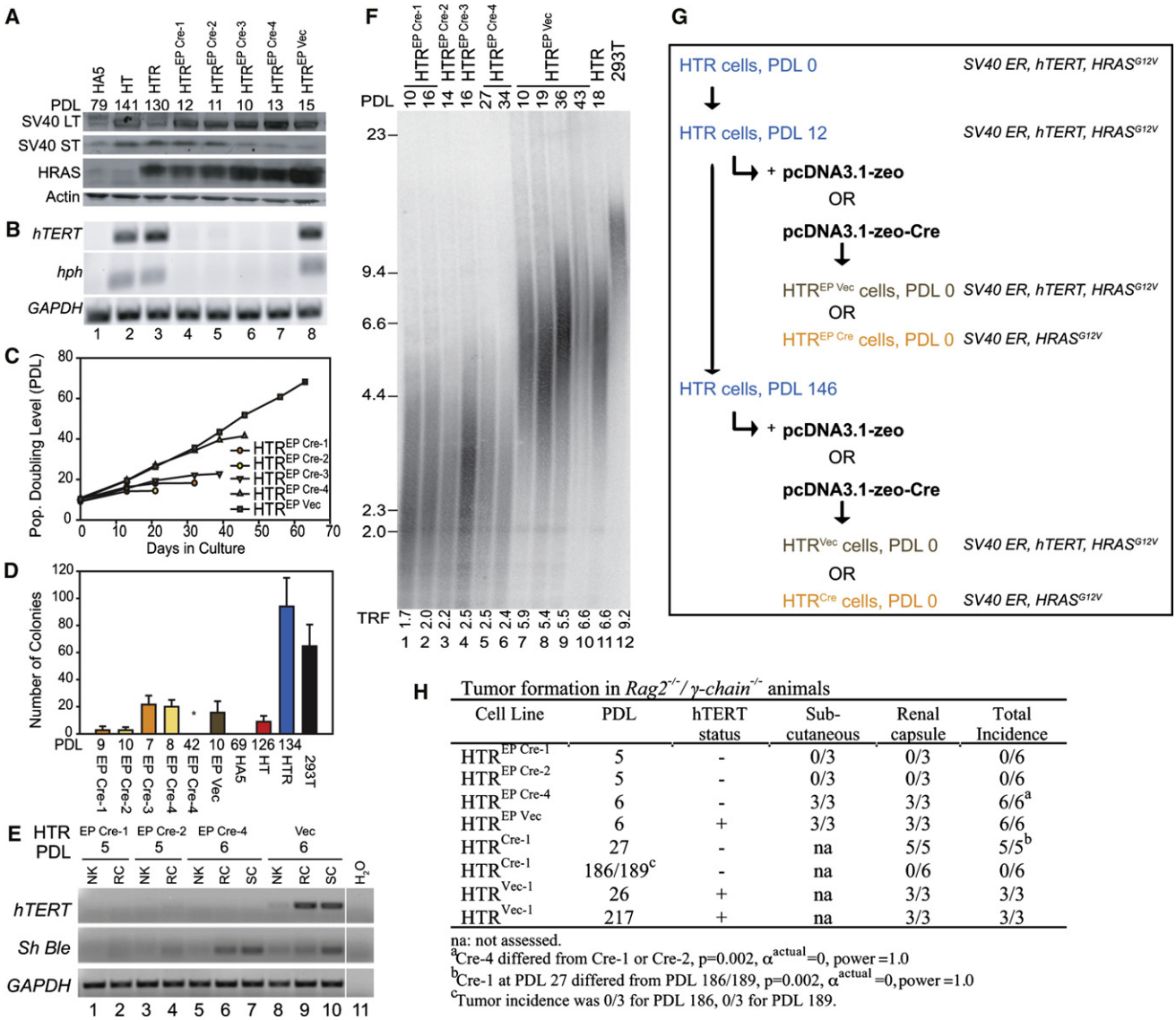
RESULTS

Establishment of hTERT-Excisable Human Tumor Cells

The human *TERT* cDNA (Harrington et al., 1997) and an *Escherichia coli* phosphotransferase gene encoding resistance to hygromycin B (*hph*) (Gritz and Davies, 1983) were flanked by loxP sites and introduced into human HA5 embryonic kidney cells (HA5) containing the SV40 early region (ER) (Stewart and Bacchetti, 1991; Figure 1). Upon *hTERT* introduction, HT (HA5 + hTERT) cells became telomerase-positive and immortal but could not support anchorage-independent growth in 0.6% w/v agar. However, after infection with a retrovirus encoding $HRAS^{G12V}$ (HT + RAS = HTR) (Hahn et al., 1999a), HTR cells formed colonies in 0.6% w/v agar and gave rise to tumors in immunocompromised mice when injected subcutaneously or beneath the kidney capsule epithelium (Figures 1 and 2H). In this tumor cell model, we chose to use an SV40-transformed cell line (HA5) that cannot escape crisis spontaneously (Counter et al., 1992), and *hTERT* was introduced as the second (rather than first) step in the tumorigenic conversion process (Elenbaas et al., 2001; Hahn et al., 1999a, 2002). Thus, immortalization is not an obligate first step for human tumorigenesis.

TERT-Excised Tumor Cells with Short Telomeres Capable of Transient Tumor Formation

After a short period of propagation in culture (population-doubling level [PDL] 12, mean TRF <6 kbp, e.g., Figure 2F, lane 11), Cre recombinase or the appropriate empty vector control encoding zeocin resistance (*Sh Ble*) was introduced into this HTR "early passage" population (HTR^{EP}) (Figures 2A and 2G), and after transient selection of clonal populations, the excision of *hTERT* (and *hph*) was queried via RT-PCR analysis (Figure 2B, lanes 4–8). Cell crisis ensued in *hTERT*-excised populations soon thereafter (Figure 2C); however, the two longest-lived cell lines supported anchorage-independent growth immediately after *hTERT* excision (Figure 2D, $HTR^{EP\ Cre\text{-}3}$ and $HTR^{EP\ Cre\text{-}4}$). $HTR^{EP\ Cre\text{-}4}$ cells, although *hTERT* negative (Figure 2E, lanes 6, 7), formed tumors in mice at an incidence indistinguishable from *hTERT*-positive HTR cells ($HTR^{EP\ Vec}$) (Figure 2H). This controlled *hTERT* genetic excision is consistent with the transient survival observed upon telomerase suppression in human cancer lines with short telomeres (Hahn et al., 1999b; Zhang et al., 1999).



H

Tumor formation in *Rag2*$^{-/-}$/*γ-chain*$^{-/-}$ animals

Cell Line	PDL	hTERT status	Sub-cutaneous	Renal capsule	Total Incidence
HTR$^{EP\ Cre-1}$	5	-	0/3	0/3	0/6
HTR$^{EP\ Cre-2}$	5	-	0/3	0/3	0/6
HTR$^{EP\ Cre-4}$	6	-	3/3	3/3	6/6[a]
HTR$^{EP\ Vec}$	6	+	3/3	3/3	6/6
HTR^{Cre-1}	27	-	na	5/5	5/5[b]
HTR^{Cre-1}	186/189[c]	-	na	0/6	0/6
HTR^{Vec-1}	26	+	na	3/3	3/3
HTR^{Vec-1}	217	+	na	3/3	3/3

na: not assessed.
[a]Cre-4 differed from Cre-1 or Cre-2, p=0.002, α^{actual}=0, power =1.0
[b]Cre-1 at PDL 27 differed from PDL 186/189, p=0.002, α^{actual} =0, power =1.0
[c]Tumor incidence was 0/3 for PDL 186, 0/3 for PDL 189.

Figure 2. Excision of *hTERT* from Tumor Cells with Short Telomeres

(A) Western analysis of cell lysates (50 μg) in HTR^{EP} (early passage) cells transfected with Cre recombinase or empty vector control at indicated population-doubling level (PDL).

(B) RT-PCR analysis of *hTERT*, *hph*, and *GAPDH* at indicated PDL.

(C) Replicative life span of indicated cell lines. HTR$^{EP\ Vec}$ remained immortal.

(D) Anchorage-independent colony formation at indicated PDL. 293T cells were included as a positive control, and HA5 as a negative control. HTR$^{EP\ Cre-4}$ at PDL 42 (no colonies) differed significantly from HTR$^{EP\ Cre-4}$ at PDL 8 (n = 3, *p < 0.05, power$^{(1-\beta\ err\ prob)}$ = 1.0, α^{actual} = 0.05, two tailed).

(E) RT-PCR analysis of *hTERT*, *Sh Ble* (zeocin) and *GAPDH* in tissue extracted from renal capsule (RC) or subcutaneous (SC) injection sites, or normal adjacent kidney (NK).

(F) TRF analysis of average telomere length at increasing PDL. Weighted mean telomere lengths (kbp) are indicated below each lane.

(G) Schematic of elements introduced into HT cells, at indicated PDL.

(H) Incidence of tumor formation of indicated cell lines in immunodeficient mice (see Experimental Procedures for details).

Error bars illustrate the standard deviation of the mean, using the number of replicates as indicated.

TERT-Excised Tumor Cells Exhibit Robust Tumor Formation until Telomere Crisis

To create *hTERT*-negative human tumor populations with long telomeres, the HTR population was propagated in culture for more than 240 days (PDL 146) until average telomere length reached 12 kbp (Figure 3E, lane 11) prior to *hTERT* excision. Control cell clones in which an empty vector (HTR^{Vec}) was introduced retained *hTERT* and *hph* expression, and exhibited telomere elongation and colony forming potential in 0.6% w/v agar (Figures 3A–3E). In clones selected for Cre recombinase

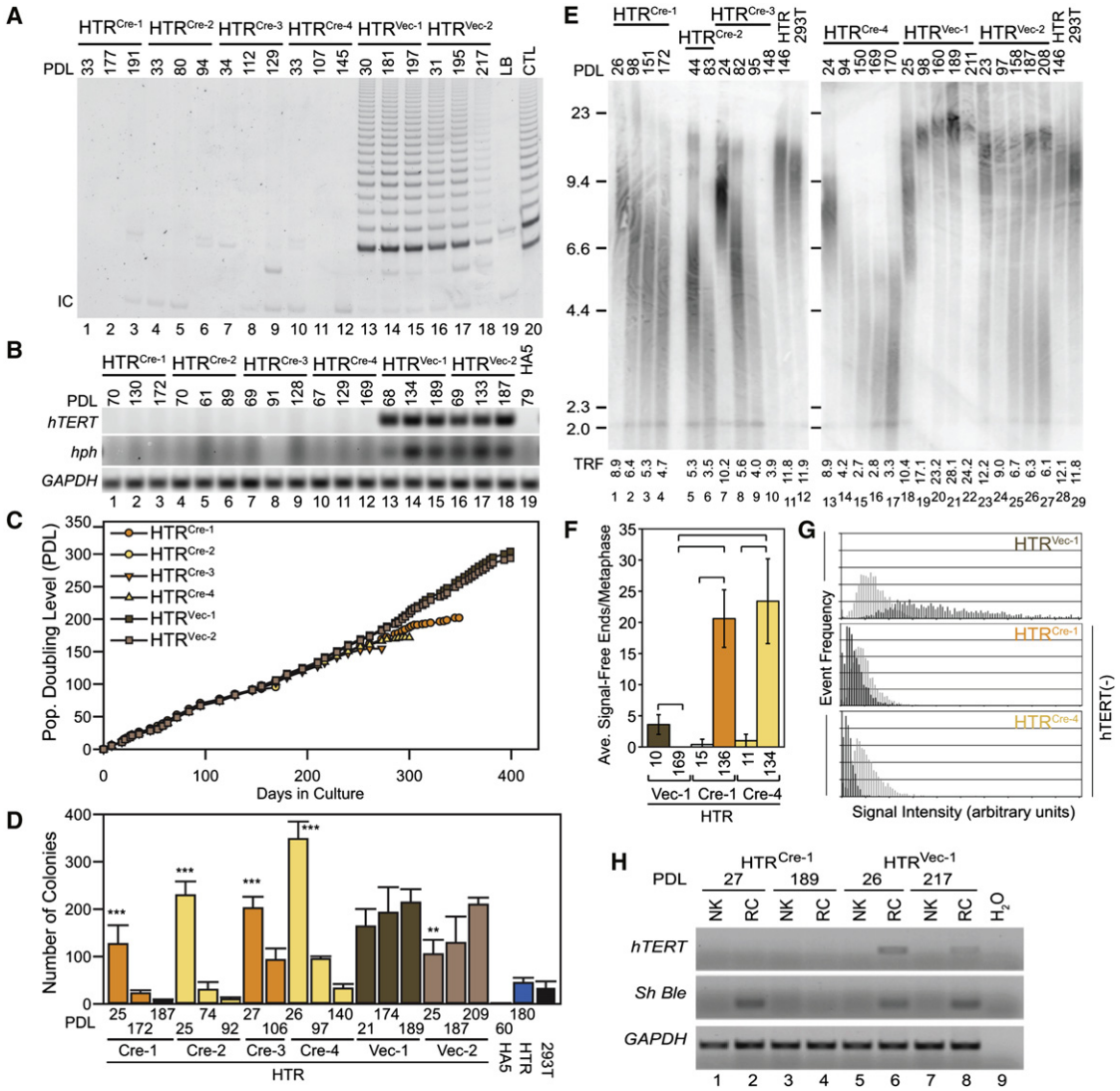


Figure 3. Excision of *hTERT* from Tumorigenic Cells with Elongated Telomeres

(A) Telomerase activity in cell lysates (200 ng) from HTR^{Cre} and HTR^{Vec} clonal cell lines at indicated PDL, controls as specified in Figure 2.

(B) RT-PCR analysis of *hTERT*, *hph*, and *GAPDH* at indicated PDL. HA5 cells were included as a negative control.

(C) Replicative life span of each clonal line, as indicated. HTR^{Vec} cells remained immortal.

(D) Anchorage-independent colony growth at increasing PDL, including HA5 and HTR cells as controls (n = 4 each), and 293T cells (n = 3). Difference between the latest and earliest PDL within each line as indicated (**p < 0.01; ***p < 0.001, power$^{(1-\beta\ err\ prob)}$ = 1.0, α^{actual} = 0.05, two tailed).

(E) TRF analysis of average telomere length at indicated PDL. Weighted mean telomere lengths (kbp) are indicated below each lane.

(F) Analysis of telomere integrity. x axis, individual lines and respective PDL; y axis, average number of telomere signal-free ends (SFE) per metaphase (n = 10). Brackets indicate a statistically significant difference (p < 0.001, power$^{(1-\beta\ err\ prob)}$ = 1.0, α^{actual} = 0.038-0.044). HTR^{Vec} at PDL 169 possessed no SFE.

(G) Relative telomere length of the lines depicted in (F). x axis, telomere fluorescence intensity in arbitrary units; y axis, frequency of events. Early PDL (light gray), late PDL (dark gray). Graphs are scaled equivalently.

(H) RT-PCR analysis of *hTERT*, *Sh Ble* (zeocin resistance) and *GAPDH* in normal adjacent kidney (NK) or renal capsule (RC). The water control (H_2O) is the same as in Figure 2E, lane 11.

Error bars illustrate the standard deviation of the mean, using the number of replicates as indicated.

expression (HTR^{Cre}), loss of *hTERT* expression was confirmed by RT-PCR and measurement of telomerase activity (Figures 3A and 3B, lanes 1–12). The maximum life span of these *hTERT*-excised clones exceeded 250 days, and one clone survived for 1 year (Figure 3C). Telomerase activity remained absent, and telomere attrition continued unabated with no evidence of the telomere length heterogeneity typical of telomerase-negative tumor cells that undergo telomere recombination (ALT) (Figures 3A, 3E, and 3G). Even in the complete absence of *hTERT*, HTR^{Cre} lines retained a significant initial capacity for anchorage-independent growth (Figure 3D). Upon injection into the subrenal capsule, which in some instances is more permissive for tumor

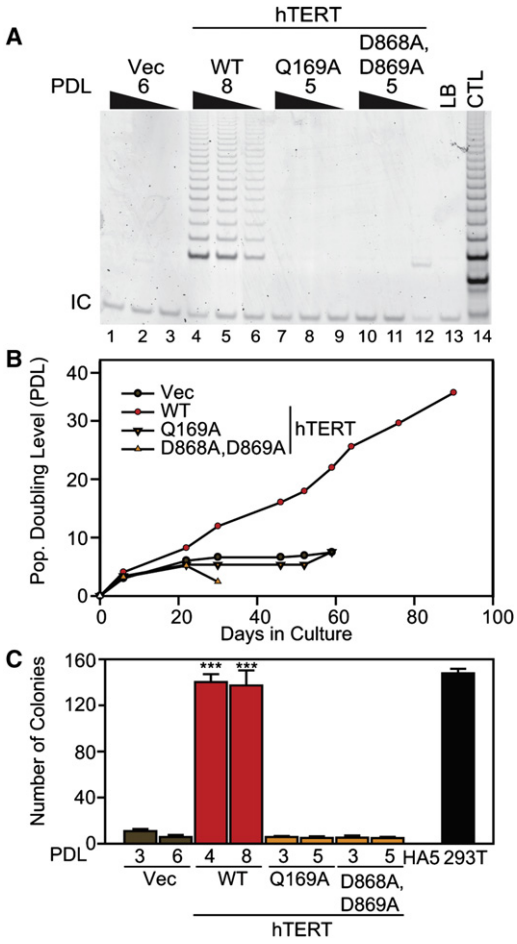


Figure 4. Ability of *hTERT* to Rescue Crisis in *hTERT*-Excised Cells

(A) Wild-type (WT) or mutant (Q169A; D868A, D869A) *hTERT* or empty vector (Vec) were introduced into $HTR^{EP\ Cre}$ cells and analyzed for telomerase activity (200, 100, 50 ng lysate).

(B) Replicative life span of cell lines as indicated above. hTERT WT cells remained immortal.

(C) Anchorage-independent growth of cell lines as indicated (n = 4). Statistical significance compared with vector controls as indicated (***$p < 0.001$, $power^{(1-\beta\ err\ prob)} = 1.0$, $\alpha^{actual} = 0.05$, two tailed). Controls and axis labels as in Figure 2.

Error bars illustrate the standard deviation of the mean, using the number of replicates as indicated.

formation than subcutaneous injection (Liang et al., 2008; Sun et al., 2005), HTR^{Cre} lines exhibited a tumor incidence of 100% even after more than 1 month in culture (PDL 27) (Figure 2H). This incidence was indistinguishable from the 100% tumor incidence of telomerase-positive HTR^{Vec} cells, and exhibited statistical significance at high probability ($\alpha = 0$, power = 1.0) compared with a tumor incidence of zero percent in HTR^{Cre} lines at late passages (PDL 186/189) (Figure 2H). Analysis of HTR^{Cre} tumor explants confirmed the absence of *hTERT* and retention of *Sh Ble* expression specific to HTR^{Cre} cells (Figure 3H, lane 2). The eventual loss of tumor-forming capability and anchorage-independent growth at later passages was coincident with the appearance of chromosome ends with no detectable telomeric DNA (Figures 2H, 3D, 3F, and 3G). These results demonstrate that longer telomere reserves permit human tumor formation for prolonged periods in the absence of telomere maintenance and *hTERT*, but that the eventual loss of telomeric DNA leads to crisis and an inability to support tumor formation.

Crisis in TERT-Excised Cells Is Rescued by Catalytically Active TERT

To confirm that crisis was induced by critically short telomeres and not via unrelated genetic events, wild-type hTERT or inactive hTERT mutants Q169A (Sealey et al., 2010; Wyatt et al., 2009) or D868A/D869A (Harrington et al., 1997) were introduced into $HTR^{EP\ Cre-4}$ cells at PDL 6 (Figure 4). Only wild-type hTERT restored telomerase activity (Figure 4A), extended cellular life span (Figure 4B), and conferred anchorage-independent growth (Figure 4C). HA5 cells without $HRAS^{G12V}$ also depend on the catalytic activity of hTERT to avoid crisis (Sealey et al., 2010). The fact that telomerase catalytic activity was essential to avert crisis and promote anchorage-independent growth supports the critical role of *hTERT*-mediated telomere extension activity in tumor cell survival when telomeres are short.

DISCUSSION

In the presence of sufficiently long telomeres, telomere erosion or the absence of *hTERT* did not impede human tumorigenesis. Only when telomeric DNA was lost from chromosome ends did cells resume dependence upon the telomere elongation activity of hTERT. Other examples of tumor-forming capability in cells that do not express *hTERT* are known, for example, in ALT cells or primary tissues transformed with oncogenic *RAS* (Liang et al., 2008; Sun et al., 2005), but these examples did not permit the ability to test the compatibility of ongoing telomere erosion with cell survival. Examples of tumors that lack in vitro telomerase activity have been correlated with clinical regression (e.g., retinoblastoma or neuroblastoma) (Gupta et al., 1996; Hiyama et al., 1995); however, these studies preceded the cloning of *hTERT* or identification of ALT and in many cases these tumor types are now known to exhibit ALT-like characteristics or low *hTERT* expression (reviewed in Cesare and Reddel, 2010). Here, we showed in a defined genetic system that telomerase-negative human tumor cells are capable of tumor formation and cell viability in the absence of endogenous *hTERT* expression or ALT.

Although tumorigenic potential has not been examined in mice lacking *Tert*, its absence has no phenotypic consequences in normal murine tissues while telomere reserves remain intact (Erdmann and Harrington, 2009; Meznikova et al., 2009; Strong et al., 2011; Vidal-Cardenas and Greider, 2010). The fact that *hTERT* is dispensable in telomerase-positive tumor cells was not foreseen. For example, deletion of one subunit of the Ku heterodimer, a complex important in maintaining telomere integrity, is lethal in human tumor cells but is dispensable in other organisms (Fattah et al., 2008; Li et al., 2002). Once telomeres became critically short, however, aversion of tumor cell crisis depended upon active TERT. In contrast, when TERT is overexpressed, its ability to stimulate proliferation does not always depend on catalytic activity, e.g., in ALT cells (Stewart et al.,

2002) or murine hair follicles (Flores et al., 2005; reviewed in Parkinson et al., 2008; Sarin et al., 2005).

Human tumor cells retained their susceptibility to telomere-induced crisis even after prolonged growth periods. This delayed dependence upon telomerase function differs from the "addiction" to oncogenic factors such as MYC or RAS, in which cell survival remains reliant on these factors (Greider, 1999; Weinstein and Joe, 2008). Thus, human tumor cells are reliant upon telomere integrity rather than *hTERT* or telomerase activity. Although such dependence was well established for normal cell growth, it was not possible to predict whether tumor cells might somehow subvert telomere-induced crisis via induction of endogenous telomerase, ALT, or another mechanism. For example, *Saccharomyces cerevisiae* lacking telomerase and the recombination factor *RAD52* can escape senescence indefinitely via activation of *RAD52*-independent telomere maintenance mechanisms, provided the strain possesses long telomeres initially (Grandin and Charbonneau, 2009; Lebel et al., 2009). In contrast, our results suggest that human tumor cells with initially long telomeres can only temporarily avert the requirement for telomere maintenance.

These results have implications for telomerase inhibition in cancer therapy. Telomerase-negative pediatric cancers such as ependymoma possess a better long-term prognosis than telomerase-positive cancers (reviewed in Tabori and Dome, 2007), and low telomerase expression or ALT correlates with a better outcome in histiocytoma and colorectal cancer (Matsuo et al., 2009; Tatsumoto et al., 2000). Our finding that telomerase-negative tumors do not invoke ALT and remain mortal may provide a mechanism to explain the more favorable prognosis for a subset of telomerase-negative tumor types in vivo. Thus, even in telomerase-positive tumors with long telomeres, telomerase inhibition combined with adjunct treatments that limit tumor progression could prove effective as an anticancer therapy.

EXPERIMENTAL PROCEDURES

Cell Culture

Cell culture and PDL determination was performed as described (Hayflick, 1973; Sealey et al., 2010). hTERT was introduced via electroporation and clonal populations selected in 200 μg/ml hygromycin (Invitrogen), followed by retroviral infection with pBABE- puro-HRAS^{G12V} (Addgene) (Hahn et al., 1999a) and selection in 2 μg/ml puromycin (Invitrogen). Transfection with pcDNA3.1-zeo-Cre (Cre recombinase cDNA provided by Dr. Michael Reth) or pcDNA3.1-zeo (Invitrogen) was performed using Fugene6 (Roche) with transient selection in 200 μg/ml zeocin (Invitrogen). Inactive hTERT variants were introduced as described (Sealey et al., 2010).

Protein and RNA Analysis

Western blots, RT-PCR mRNA analysis, and the telomere repeat amplification protocol (TRAPeze, Millipore) were performed as described (Sealey et al., 2010). RT-PCR analysis of *mRNA* encoding zeocin resistance (*Sh Ble*) was conducted using the following DNA primers: 5′-GACTTCGTGGAGGACGACTT-3′ and 5′-GACACGACCTCCGACCACT-3′. Primary antibodies employed were anti-SV40 T Ag (Pab-108) (Santa Cruz), anti-HRAS (C-20) (Santa Cruz), and anti-actin (Sigma).

Anchorage-Independent Growth Assay

Equal cell numbers (5×10^4) were plated onto 0.6% w/v agar and incubated at 37°C (5% v/v CO_2) for 21 days as described (Cifone and Fidler, 1980). Colonies were stained with 0.01% w/v crystal violet and images acquired with a Bio-Rad Molecular Imager Gel Doc XR System. Colonies were counted using Imagequant TL (GE Healthcare).

Cell Line Injections In Vivo

A suspension of 5×10^5 cells was injected subcutaneously or under the subrenal capsule space of *Rag2*$^{-/-}$ / γ-*chain* $^{-/-}$ immunodeficient mice (Mazurier et al., 1999). After 21–22 days, the mice were sacrificed and examined. Explanted tissues were extracted for RNA and analyzed by RT-PCR as described above. Experiments were performed in accordance with protocols approved by the Animal Care Committee at the University of Guelph, as outlined in the animal utilization protocol AUP08R007 (issued to D.H.B).

Telomere Terminal Restriction Fragment and Q-FISH Analysis

Telomere length was analyzed via terminal restriction fragment (TRF) analysis (Sealey et al., 2010), and average length determined after Southern blotting using Imagequant TL and UTSWTELORUN software first developed by H. Vaziri and C. Harley (Ouellette et al., 2000). Q-FISH was performed as described (Erdmann and Harrington, 2009) on ten separate metaphases for each PDL indicated.

Statistical Analysis

Differences in average colony number were assessed via analysis of variance (ANOVA), assuming unequal variance and using a Tukey post-test (Instat3, GraphPad). Statistical significance of tumor incidence was assessed using Fisher's exact test (Prism5, GraphPad). G*power3 was used to determine power and alpha values where indicated (Faul et al., 2009). Quantification of telomere-signal free ends (SFE) after Q-FISH was compared using ANOVA with a Tukey post-test (Instat3, GraphPad).

LICENSING INFORMATION

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